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Comparing Flow Cytometry and Fluorescence Microscopy for Analyzing Human Sperm DNA Fragmentation by TUNEL Labeling

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• **Abstract**

Conflicting results are reported by recent studies comparing flow cytometry (FCM) and fluorescence microscopy (FM) for detecting sperm DNA fragmentation by TUNEL assay. Each of the two technologies has specific peculiarities and limitations, but whereas the limitations of FM observation are well known, the biases due to the inability of FCM to recognize morphologically analyzed cells are less explored. In particular, so far, FCM analysis of sperm DNA fragmentation have included in the analyses M540 bodies, round semen structures exhibiting FSC/SSC properties similar to sperm. Semen M540 bodies, altogether with the occurrence of two sperm populations with different nuclear staining, concur to an underestimation of values of sperm DNA fragmentation by FCM. However, even considering such bias, the observed discrepancies between the performance of FM and FCM are not fully explained. We discuss here the possible variables that may affect the results of each of the two technologies and the necessary efforts to be taken to address this issue.

• **Key terms**

sperm; DNA fragmentation; Tunel; flow cytometry

WE read with great interest the paper "Human Sperm DNA Fragmentation: Correlation of TUNEL Results as Assessed by Flow Cytometry and Optical Microscopy" by Domínguez-Fandos et al., in the 71A issue of Cytometry Part A 2007 (1). This study addresses the important problem of comparing measures of sperm DNA fragmentation obtained by revealing TUNEL labeling both by flow cytometry (FCM) and fluorescence microscopy (FM) in the same semen samples ($n = 66$). They found that FCM yields values of sperm damage that are 2.6 times greater than those obtained by FM. Such value seems enough in agreement with the ratio (1.6) obtained in a previous study by our group (2) conducted, however, in swim-up selected sperm and in a smaller number of subjects ($n = 13$). In contrast, in a recent investigation (3), exploring the same issue in 68 subjects, the values of sperm DNA fragmentation obtained by FM resulted much greater than those obtained by FCM.

To compare sperm TUNEL positivity between FCM and FM, the intrinsic and specific differences between a flow cytometer and a microscope have to be considered. The peculiarities of each of the two technologies are well known (4). The main advantage of revealing fluorescence by FCM consists in the possibility to perform a large number of measures in the test sample, in an objective, rapid, and reproducible manner. It is also current opinion that FCM is more sensitive than FM, at least when the latter relies on human eye for fluorescence detection. In addition, the duration of observation in FM is necessarily longer than in FCM, thus possibly contributing to neglect a fraction of positive cells due to fluorescence bleaching. On the other hand, the great disadvantage of FCM is the fact that it cannot directly recognize the structures emitting fluorescence, unlike in optical microscopy. This limitation is only partially overcome by the gating procedure, i.e. by drawing boundaries around subsets of events in data plots. For instance, gating clusters of events in the FSC/SSC dot plots may be a rough procedure especially when a complex and heterogeneous sample, such as seminal fluid, is examined. In

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human semen there can be both somatic and germ cells with various degree of maturity, other than cell debris. In addition, in recent years, it has been also reported the occurrence of round semen structures, termed M540 bodies, due to their prompt staining with Merocyanine 540 and containing small quantities of fragmented DNA (for the nature and origin of M540 bodies, the reader is referred to Refs. 5 and 6). M540 bodies occur in high level especially in semen of subfertile patients (5,6) and are partially located in the same FSC/SSC region containing sperm (5–7), representing an important interference in flow cytometric analyses of sperm. In particular, M540 bodies, if not excluded from the analyses, concur to underestimate the percentages of TUNEL positivity (8) in most semen samples. When data are processed by setting a threshold in the negative control (including more than 99% of the events) and translating such threshold in the corresponding test sample, M540 bodies may interfere with calculation of sperm DNA fragmentation for two main reasons. First, they may have a level of autofluorescence greater than that of sperm and thus shift the threshold setting toward the high values of DNA fragmentation in the negative control (see, for instance, Fig. 2 of Ref. 8). As a consequence, a fraction of DNA fragmented sperm is masked and neglected. Second, the percentage of M540 bodies with TUNEL positivity is low (6), and thus most of them (TUNEL negative) contribute to increase the percentage of global TUNEL negative events (8). Up to now, the easiest way to exclude M540 bodies from the cytometric analysis is to stain the samples with a nuclear probe, as propidium iodide (PI). Indeed, as M540 bodies are devoid of nucleus (5), they do not label (or poorly label) with nuclear dyes (5–8) and can be easily subtracted from the FSC/SSC region of sperm (7,8). In the past, PI staining has been used in some studies on sperm DNA fragmentation by TUNEL coupled to FCM (9,10), including that by Domínguez-Fandos et al. (1) and a previous study from our group (2). However, in these studies such staining is used to back gate the PI-positive events in the FSC/SSC dot plot (i.e. to localize PI-positive events in the FSC/SSC dot plot) to better define the region of the population of interest. Such procedure is not adequate to exclude M540 bodies from the analysis since they are partially located within such region, thus affecting the results. Conversely, M540 bodies should not have any influence on sperm DNA fragmentation assessment by FM because they are easily distinguishable from sperm.

In FCM, PI staining of sperm samples also allows to discriminate two sperm populations, one more (PI brighter) and one less (PI dimmer) labeled with such nuclear dye (8). PI dimmer population may exhibit a level of autofluorescence greater than PI brighter sperm, in some subjects (see Fig. 2 of Ref. 8). Hence, the lack of such discrimination can yield a cytometric underestimation of DNA fragmentation in the PI brighter sperm (8). Although the occurrence of the two sperm populations, and in particular their different autofluorescence, may affect the measures in FCM, it is not known if and how they interfere with the optical determinations. In the latter case, it is important to note that optical observation is not able to rigorously distinguish the difference of the quantity of PI fluorescence emitted by two sperm subpopulations (Muratori et al., unpublished data).

In light of the occurrence of M540 bodies in semen, we can discuss results of the three studies directly comparing TUNEL sperm detection by FCM and FM. Apparently, the lack of exclusion of semen M540 bodies from sperm analysis might explain the greater values yielded by FM respect to FCM in the study of Cohen-Bacrie et al. (3). On the other hand, the fact that in the study of Domínguez-Fandos et al. (1) M540 bodies were not excluded from the analysis suggests that the observed FCM/FM ratio might be even greater than that they reported (2.6). Further, the difference between the ratio observed by Domínguez-Fandos et al. (1) and that (1.6) calculated in our previous study (2) in swim-up selected sperm might be greater as well. Indeed, the presence of M540 bodies (not yet described at that time) is reduced after swim-up selection (5), and thus they should interfere less with the measures of TUNEL coupled to FCM.

From the comparison of these few investigations on sperm TUNEL results obtained by FCM and FM, it appears evident that the specific peculiarities of FCM and FM are not sufficient to explain and discuss their different performance in detecting sperm TUNEL labeling. Indeed, it is important also to consider that different procedures of TUNEL assay coupled to both FCM and FM can be performed, even when the same protocol to label sperm is used. Microscopic determination of TUNEL-positive spermatozoa is affected by factors such as the subjectivity and the duration of the observations (because of fluorescence bleaching), both difficult to standardize. On the other hand, as thoroughly discussed by Domínguez-Fandos et al. (1), other factors affect the measures obtained by FCM because different procedures exist to establish the population of interest, to gate it, and to estimate the amount of positive events. The latter issue appears an important source of variability in calculating sperm DNA fragmentation by FCM. The three mentioned studies used a similar method of threshold setting to determine the percentage of TUNEL-positive sperm, but on different reference samples. Cohen-Bacrie et al. (3) set a threshold in the positive sample (i.e. treated with external nucleases) including >90% events; we (2) and Domínguez-Fandos et al. (1), established a threshold including >99% events in the negative control. It is important to note that even the same method to set the threshold between negative and positive events does not guarantee the same result. Indeed, the distinction between fragmented and not fragmented DNA cells in the sperm sample is not sharp and the distribution of fluorescence intensities in test sample very often overlaps that of autofluorescence. Hence, even a very little shift in threshold setting in negative control may produce a great difference in positivity of the test sample.

In our opinion, future efforts should focus on increasing the number of investigations directly comparing FCM and FM, on standardizing the procedure of TUNEL assay, and on further investigating which part of discrepancy between FM and FCM can be ascribed to the specific peculiarities of the two systems.

In this context, much help can be provided by other technologies able to virtually overcome all of the mentioned lim-

itations in detecting cell fluorescence by both FM and FCM, by combining the higher throughput of FCM with ability of FM to directly visualize the analyzed cells. Among such technologies, systems able to acquire integrated fluorescence signals and high-quality images from large numbers of cells in flow (11) are emerging, in addition to the well-established laser scanning cytometry (12). The latter has been recently employed to automate FISH scoring in sperm, with a drastic shortening of the time necessary for manual score by FM (13).

The ability to visualize the analyzed cells should allow to directly recognize sperm from other components of semen, including M540 bodies, and to increase the specificity of FCM analysis. In particular, it could be verified whether false TUNEL-positive sperm are counted by traditional FCM, as already reported in somatic cells, due to the adhesion of TUNEL-positive DNA fragments to cell surface (11). The occurrence of false-positive TUNEL events altogether with a lower sensitivity of FM could represent other elements to explain why FCM yields so greater measures of sperm DNA fragmentation with respect to FM, even when the same labeling method and a very similar procedure to count positive sperm versus the negative ones are used. Indeed, to set a threshold including virtually any fluorescence intensity of the FCM negative control [as performed in some studies (1,2)] strictly resembles the way to proceed in FM, where the threshold is that of human eye.

In conclusion, the comparison between sperm TUNEL measures from FCM and FM is still an open matter. Whereas the limitations due to subjectivity and less sensitivity are generally assumed for FM measurement, we report here that biases of FCM assessment due to its inability to visualize analyzed cells occurred so far. They consist in the presence of (i) nonsperm elements (such as M540 bodies), (ii) false-negative sperm (such as the fraction of brighter sperm masked by the autofluorescence of the dimmer ones, in certain subjects), and possibly, false TUNEL-positive sperm.

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